Vinorelbine Potently Induces Placental Cell Death, Does Not Harm Fertility and is a Potential Treatment for Ectopic Pregnancy

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Research Paper

Vinorelbine Potently Induces Placental Cell Death, Does Not Harm Fertility and is a Potential Treatment for Ectopic Pregnancy

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ABSTRACT

Ectopic pregnancies complicate 1–2 pregnancies and are a leading cause of maternal death. An effective oral drug therapy that replaces surgery might make its treatment safer, cheaper, simpler and therefore more widely accessible. The only current medical treatment offered to women is intramuscular methotrexate, but this only reliably resolves smaller ectopic pregnancies. As such, many ectopic pregnancies require surgical excision. We show that vinorelbine, an orally available chemotherapeutic agent, potently induced placental cell death but did not harm fertility in mice. Vinorelbine was 100–1000 times more potent than methotrexate in inducing placental cell death in vitro, and more potent than combination methotrexate and gefitinib (another proposed treatment for ectopic pregnancy being evaluated in phase III trials). Mechanistically, it caused microtubule condensation, blocked mitosis and activated the apoptosis cascade in placental cells. Vinorelbine was more efficacious than methotrexate ± gefitinib in reducing the volume of placental cell tumors xenografted subcutaneously in SCID mice. Mice exposed to vinorelbine and allowed to breed, following a four week washout period, displayed normal fertility, however long-term fertility was not assessed. Human Fallopian tubes treated with vinorelbine did not exhibit up-regulation of apoptosis molecules. Our findings show that placental cells appear sensitive to vinorelbine and it has potential as a tablet-only approach to treat ectopic pregnancy.

1. Introduction

Ectopic pregnancies arise when a conceptus implants outside the uterine cavity, with over 98% implanting in the Fallopian tube (Bouyer et al., 2002). They are life-threatening as they can erode through maternal vessels and cause fatal bleeding (Knight et al., 2016) and are one of the main causes of maternal death during the first trimester. They are also common, complicating 1–2% of all pregnancies with around 100,000 cases diagnosed each year in the United States alone (Centre for Disease Control and Prevention, 1995).

Many are treated surgically (Jurkovic and Wilkinson, 2011) where often, the entire Fallopian tube is removed together with the ectopic pregnancy. A simple and highly efficacious medical option could make the treatment of ectopic pregnancy safer (avoiding the risks of surgery), simpler (requiring less highly trained staff) and cheaper. The further implications are that the treatment could become more accessible in resource poor settings where surgery is either difficult to access, or not available.

There is one medical treatment offered clinically instead of surgery, intramuscular methotrexate (RCOG, 2016; The ACOG Task Force, 2008). However, methotrexate is only efficacious for smaller ectopic pregnancies that fulfill strict clinical criteria, and its rates of failure are too high for larger ectopic pregnancies (which will then often require salvage surgery) (RCOG, 2016). Furthermore, it often takes approximately one month for the ectopic pregnancy to resolve following methotrexate management (Skubisz et al., 2013) and, owing to its limited effectiveness, is less cost effective than laparoscopic (surgical) excision(Mol et al., 2008). Consequently, many ectopic pregnancies are surgically excised (Jurkovic and Wilkinson, 2011).

We previously identified the possibility that adding gefitinib tablets (an epidermal growth factor inhibitor that adversely affects placental signaling to cause apoptosis) to the intramuscular methotrexate might

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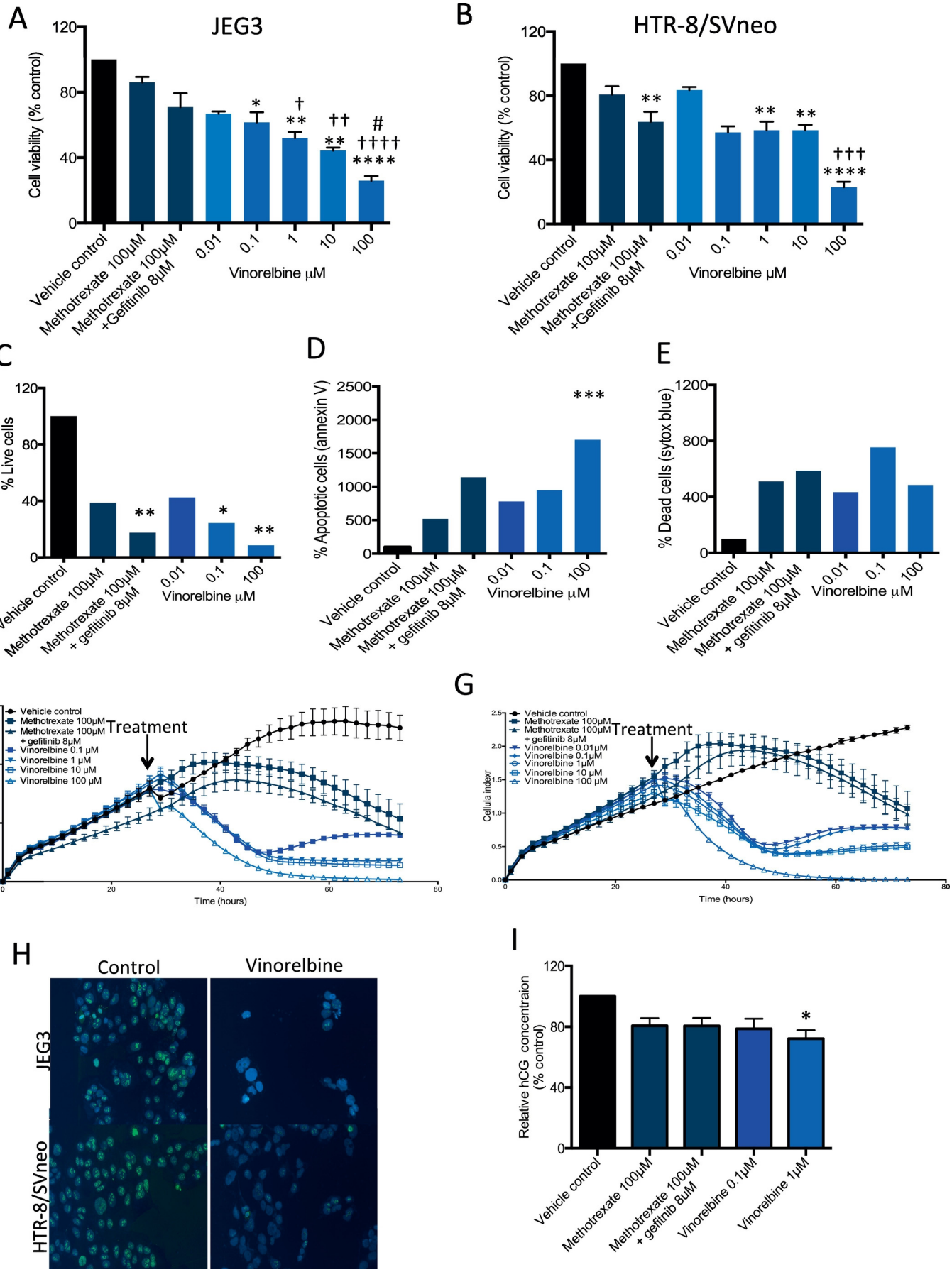

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enhance its efficacy (Skubisz et al., 2013; Nilsson et al., 2013), and we are currently evaluating this in a phase III randomized trial (EudraCT No: 2015-005013-76). Aside from this, there appears to be a distinct lack of other medical alternatives to treat ectopic pregnancy, either touted at the preclinical stage, or in trials (Tong et al., 2015).

Vinorelbine is a semi-synthetic vinca-alkaloid derived from the Madagascar periwinkle (Noble, 1990). First manufactured in 1979, it is considered a well tolerated chemotherapeutic. Vinorelbine induces cytotoxicity by binding to tubulin, which disrupts mitotic spindle formation and results in arrest of the cell cycle at the metaphase to anaphase transition. This ultimately leads to apoptosis of mitotic cells (Wang et al., 1999). Vinorelbine is highly selective to mitotic rather than axonal microtubules, which means it preferentially targets dividing and highly proliferative cells (Binet et al., 1989; Paintrand and Pignot, 1983). It is currently approved to treat non-small cell lung cancer, Hodgkin's lymphoma, breast and ovarian cancers (Gregory and Smith, 2000). Given the placenta is highly proliferative during the first trimester but the Fallopian tube is not, it is plausible that vinorelbine could be efficacious in resolving an ectopic pregnancy whilst minimally affecting the surrounding tube.

Importantly, there is an oral formulation of vinorelbine. Thus, if found to be highly effective the potential exists that a tablet-only treatment could be used in place of surgery to treat most ectopic pregnancies. Here, we set out to investigate the potential of vinorelbine to treat ectopic pregnancy. We compared the efficacy of vinorelbine to methotrexate, and to combination methotrexate and gefitinib.

2. Materials and Methods

2.1. Cell Culture and Treatment

Trophoblast cell lines, JEG3 and HTR 8/SVneo were cultured at 37 °C in 5% CO_2 humidified atmosphere in DMEM (Life Technologies, California, USA) or RPMI (Life Technologies) medium supplemented with 10% fetal bovine serum (FBS). For various experiments, cells were treated with the following drugs at the dose ranges of 0.01–100 μM vinorelbine (Sigma, Croydon, UK), vincristine (Sigma), etoposide (Sigma), gemcitabine (Sigma), 5-fluorouracil (Sigma), cisplatin (Sigma), herceptin (Roche, Basel, Switzerland), Actinomysin D (Sigma) or vehicle control. For some experiments we also treated drugs with gefitinib (Sigma) at 8 μl. All experiments were repeated at least three times in triplicate.

2.2. In Vitro Cell Viability and Proliferation

Cell viability was measured via MTS assay 48 h after treatment, as per manufacture's instructions (Promega, Wisconsin, USA) optical density determined using BioRad X-Mark microplate spectrophotometer (BioRad, California, USA).

Evaluation of cellular proliferation was measured using the real-time monitoring system xCELLigence E-Plate 96 according to manufacturer's instructions (Roche diagnostics, New South Wales, Australia). Cells were treated for 24 h after seeding and electrical impedance measured to derive cell index, providing a real time representation of cell growth. Measurements were recorded every 2 min for the first 2 h and every hour thereafter. Data were analyzed using RTCA software (Roche).

2.3. Immunofluorescence

After treating JEG3 and HTR-8/SVneo cells for 4 or 16 h with vinorelbine, cells were fixed in 4% paraformaldehyde for 30 min at room temperature and permeabilized with triton X-100 (Sigma) for two minutes. Primary antibodies Ki67 (anti-rabbit; 1:1000; Abcam, Cambridge, UK) or α-tubulin (anti-rabbit; 1:250; Millipore, Massachusetts, USA) were diluted in 1%BSA with 0.05% Triton X100 and applied at room temperature overnight, followed by application of Alexafluor488 fluorescently conjugated secondary antibody (anti-rabbit; 1:1000; Invitrogen, California, USA) for 1 h at room temperature. Cells were mounted onto coverslips using mounting medium containing DAPI (Sigma). For Ki67 staining images were captured using the EVOS-FL microscope. α-tubulin staining was visualized using confocal microscopy outlined below.

2.4. Confocal Microscopy

Fixed-cell confocal microscopy was performed using a Zeiss 880 laser scanning confocal microscope (Carl Zeiss, North Ryde, Australia). α-Tubulin staining was excited using 488 nm laser and emission was detected between 493 and 630 nm with standard fluorescence detector (multi alkali PMT). Nuclear staining (DAPI) was excited with 405 nm and emission was detected between 410 and 502 nm. The resulting fluorescence was imaged using 63x oil objective (NA 1.4) and acquired digitally with pixel size of 71 nm in x and y. Images were taken as Z stack with 400 nm interval and maximum intensity projection images were created for 2 dimensional view. Figures were prepared using image J software (NIH).

2.5. Flow Cytometry

To assess apoptosis and viability HTR-8/SVneo cells were labeled with APC-annexin V (BD Pharmingen, New Jersey, USA) and Sytox Blue (Life Technologies) in buffer containing 140 mM NaCl, 2.5 mM $CaCl_2$, and 10 mM HEPES in water, pH 7.4 after treating for 24 h. Flow cytometry was performed with the use of a FACSVerse flow cytometer (BD Pharmingen), using the following excitation lasers and [band pass detection filters]: APC-annexin V, 640 nm [660/10], Sytox Blue, 405 nm [448/45]. Compensation controls consisted of 1) unstained cells, 2) APC-annexin V-labeled cells, and 3) Sytox Blue–labeled cells. Flow cytometry data were analyzed with the use of FlowJo software (v7.6.4, Tree Star, Inc., Ashland, Oregon) as follows. Plots were displayed with side scatter vs. forward scatter and gated to remove debris. Plots were then displayed with Sytox Blue vs. APC-annexinV and separated into quadrants. From this plot, the percentage of live (APC-annexin V^{low}/Sytox $Blue^{low}$), percentage of apoptotic (APC-annexin V^{high}/Sytox $Blue^{low}$), and percentage of dead (Sytox $Blue^{high}$) cells were determined.

Fig. 1. Vinorelbine reduces placental cell viability and proliferation. (a) JEG3 and (b) HTR-8/SVneo cell viability measured via MTS assay after treating for 48 h treatment. Vinorelbine dose dependently reduced JEG3 and HTR-8/SVneo viability, whilst combination methotrexate and gefitinib reduced HTR-8/SVneo viability. Vinorelbine at 1, 10 and 100 μM reduced JEG3 viability beyond that seen for methotrexate 100 μM methotrexate and in HTR-8/SVneo cells at 100 μM. Vinorelbine at 100 μM in both trophoblast cell types significantly reduced viability beyond the combination methotrexate and gefitinib. Data are means ± SEM of six independent experiments in triplicate. (C–E) FACs cell sorting used to measure proportion of (C) live, (D) apoptotic and (E) dead HTR-8/SVneo cells after indicated treatments. Vinorelbine alone at 0.1 and 100 μM, and combination methotrexate and gefitinib reduced the proportion of live cells relative to vehicle control (C). The proportion of apoptotic HTR-8/SVneo cells increased with 100 μM vinorelbine (d). Data are means ± SEM of three independent experiments in triplicate. (F) JEG3 and (G) HTR-8/SVneo proliferation measured in real-time via xCELLigence. Vinorelbine significantly reduced JEG3 and HTR-8/SVneo proliferation at all doses, 0.01–100 μM, within 10 h of treating. Methotrexate and combination methotrexate and gefitinib did not significantly reduce proliferation at any time point in either cell type. Data are means ± SEM over time, of three independent experiments in triplicate. (H) Representative images of immunofluorescence showing a reduction in the proliferative marker ki67 Ki67 (in green) after treating JEG3 and HTR-8/SVneo cells with 1 μM vinorelbine (right hand panels) or equivalent control (left hand panels) for 4 h. Blue staining represents DAPI nuclear staining. (I) First trimester placental explants treated for 48 h with methotrexate, combination gefitinib and methotrexate, vinorelbine or vehicle control. Vinorelbine at 1 μM reduced secretion of β-hCG relative to control. Data are means ± SEM of five independent patient samples in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. vehicle control. †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$, ††††$p < 0.0001$ vs. methotrexate. #$p < 0.05$ vs. combination methotrexate and gefitinib. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Proportions of HTR-8/SVneo cells in the cell cycle phases were determined, after treating for 12 h, via DNA staining with propidium iodide (Sigma). Cells were fixed with 70% ethanol at −20 °C for 10 min followed by staining with 50 μg/ml propidum iodide in PBS containing 1 mg/ml RNase A (Sigma) for 30 min. Flow cytometry for propidium iodide was performed using 488 nm excitation and a 586/42 emission filter. Data were displayed with side scatter vs. forward scatter and gated to remove debris, and then as histograms for propidium iodide intensity on a linear scale. The FlowJo CellCycle algorithm with univariate modeling was used to segment plots into G_0/G_1, S, and G_2/M phases of the cell cycle and to calculate the percentage of cells in each segment.

2.6. Ex Vivo Explants

First trimester placental tissue (10–11 + 4-wk gestation; n = 5) were collected from consenting non-smoking women aged between 22 and 39 (mean: 31) years with a body mass index (BMI) below 31, undergoing elective termination of pregnancy (Patient characteristics Table. S1). Placental tissue was cut into small pieces and cultured in DMEM (Life Technologies) media containing 10% fetal bovine serum (FBS) at 37 °C in 2% O_2 overnight. Explants were treated in triplicate with vinorelbine; 0.1 and 1 μM, methotrexate; 100 μM, combination methotrexate and gefitinib; 100 μM and 8 μM, respectively, or vehicle control for 48 h. Tissue was weighed and culture supernatant collected and stored at −20 °C for subsequent β-hCG assay.

Human fallopian tubes were collected at the time of hysterectomy from non-smoking pre-menopausal women aged between 29 and 50 (mean 42) and BMI below 27 (Patient characteristics Table. S1). Tissue was collected from the ampullary region of the fallopian tube, cut into small pieces (2–3 mm) and cultured in RPMI (Life Technologies) media containing 10% FBS at 37 °C in 5% O_2 for 1 h. Explants were then treated with increasing doses of vinorelbine (0.01, 0.1, 1 and 100 μM) or vehicle (PBS) control for 24 h (in triplicate). Explants were collected and fixed in RNAlater (Life technologies) for 48 h then subject to western blot analysis. Human Ethics approval was obtained for this study from the Mercy Health Human Research Ethics Committee (R11/34) and Lothian Research Ethics Committee (study code LREC 08/S1101/1), and women gave informed written consent.

2.7. Western Blots

Cell and tissue lysates were prepared on ice in RIPA buffer (25 mM tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1% Pierce protease and phosphatase inhibitor), followed by homogenization and then centrifugation at 20,000 *g* for 10 min at 4 °C to remove cell/tissue debris. SDS–polyacrylamide gel electrophoresis (12 and 15%) with transfer onto PVDF membranes was then performed with 15 μg (cells) or 20 μg (tissue) of protein loaded per a sample. After blocking, membranes were incubated with the primary antibody overnight at 4 °C. Dilutions were as follows: anti-caspase-9 (Cell Signaling Technology, Massachusetts, USA), 1:1000; anti-caspase-3 (Cell Signaling Technology), 1:1000; anti-Bcl-2 (Cell Signaling Technology), 1:1000; anti-BAX (Abcam), 1:2000; anti-BNIP3 (Sigma), 1:500; anti-ctyochrome-c (Abcam), 1:250; anti-GAPDH, 1:5000. Anti-rabbit and anti-mouse secondary antibodies were used at 1:2500 and 1:10,000, respectively. Targeted protein signal was detected using an ECL detection kit (Amersham Bioscience) and captured with ChemiDoc imaging system (BioRad). Images were analyzed using ImageJ software (version 1.50i).

2.8. Mouse Xenografts and Treatment

All procedures were conducted under an animal care and use protocol, approved by the Austin Animal Ethics Committee at the Austin Hospital. 1×10^6 JEG3 cells were subcutaneously injected into the right flank of 7–8 week old female SCID mice. Once palpable tumors had formed mice were randomized into treatment groups and intravenous treatment commenced via tail-vein injection, with three treatments over a two-week period. Treatment groups consisted of (i) control, phosphate buffer solution (PBS); (ii) vinorelbine 1.25 mg/kg; (iii) vinorelbine 2.5 mg/kg; and (iv) vinorelbine 5 mg/kg. Following initial dose finding vinorelbine treatments, a second group of mice were treated as follows; (i) control, PBS + DMSO; (ii) methotrexate 2 mg/kg + DMSO; (iii) methotrexate 2 mg/kg + gefitinib 25 mg/kg and (iv) vinorelbine 2.5 mg/kg + PBS + DMSO. In a third set of experiments, mice were treated with methotrexate alone at the doses of 0.625, 1.25 and 2.5 mg/kg, or vehicle control on days 6 and 9 post JEG3 inoculation. Mice were monitored and tumor volume recorded every second day for up to eight days. At the conclusion all mice were euthanized, blood collected via cardiac puncture and tumors removed and weighed. Murine blood was centrifuged at 1500 *g* for 10 min, and serum stored at −20 °C for subsequent β-hCG assay. Note, in accordance with animal ethics requirements, mice were humanely killed once tumor volume rose above 1000 mm^3.

2.9. Mice Fertility Studies

Female 6–8 week old Swiss mice were treated intravenously with control PBS or vinorelbine 5 mg/kg, three times over two weeks. Following the last treatment mice were rested for four weeks and subsequently mated with 6–8 week old Swiss male mice. At embryonic day 17.5 pregnant mice were euthanized; pups and placenta collected, weighed, measured and fixed in paraformaldehyde for 24 h. Maternal blood was collected via cardiac puncture, centrifuged at 1500 ×*g* for 10 min, and serum stored at −80 °C for subsequent Anti-Mullerian Hormone (AMH) assay.

2.10. ELISA

Human β-hCG (Alpco, United States) concentration was measured in cell culture medium taken from first trimester placental explants and serum collected from xenograft mice at the time death. Mouse AMH (Elabscience, United States) concentrations were measured in serum from mice where breeding post-vinorelbine treatment was assessed. Samples were assessed in duplicate using commercial ELISA kits according to manufacturer's instructions.

2.11. Statistics

The data were analyzed for statistical significance using Graph Pad Prism 6 (GraphPad Software, La Jolla, CA). Data was tested for normal distribution and statistically analyzed as appropriate. When three or more groups were compared a 1-way ANOVA (for parametric data) or Kruskal-Wallis test (for non-parametric data) was used. Post-hoc analysis was carried out using either the Tukey (parametric) or Dunn's test (non-parametric). When two groups were analyzed, either an unpaired *t*-test (parametric) or a Mann-Whitney test (non-parametric) was used. All data is expressed as mean ± SEM. P-values < 0.05 were considered significant.

Fig. 2. Vinorelbine resolves placental mass in xenograft JEG3 model. JEG3 cells were subcutaneously injected into female SCID mice, and treatment administered intravenously. All mice were treated (indicated by ↓) three times over two weeks and tumor volume measured every second day. (A-D) Mice treated with increasing doses of vinorelbine, 1.25 mg/kg, 2.5 mg/kg, 5 mg/kg, or equivalent PBS (n = 8 per group). (A) Tumor volume (mm^3) over time and (B) tumor volume at time of death is reduced with increasing doses of vinorelbine. (C) Vinorelbine dose dependently reduced tumor weight and (D) serum β-hCG levels at time of death. Data are means ± SEM. *p < 0.05, **p < 0.01, **p < 0.001. (e–h) Mice treated with vinorelbine 2.5 mg/kg, methotrexate 2 mg/kg, methotrexate 2 mg/kg + gefitinib 25 mg/kg, or vehicle control (n = 6 per group). (E) Vinorelbine alone reduced tumor volume over time and (F) at time of death compared to vehicle control, methotrexate and combination methotrexate and gefitinib. (G) Tumor weight is reduced by vinorelbine compared to control and methotrexate. (H) Vinorelbine reduced serum hCG levels below all other treatment groups. Data are means ± SEM.

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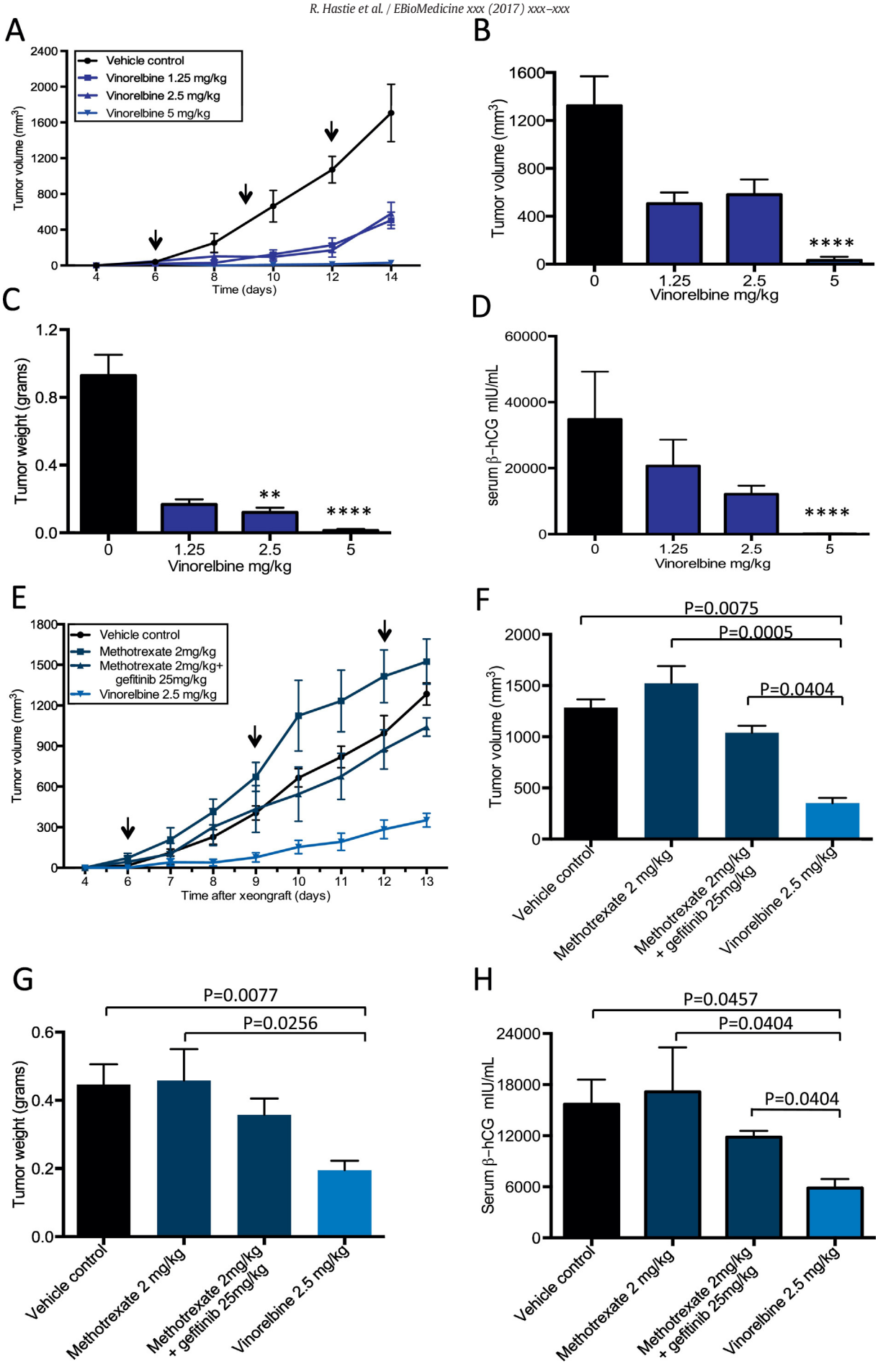

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3. Results

3.1. Vinorelbine is a Potent Inducer of Placental Cell Death

It is likely that methotrexate resolves ectopic pregnancies by inducing cell death within the placenta. Thus, drugs that induce placental cell death may be effective in medically treating ectopic pregnancies. We initially screened eight chemotherapeutic agents, by MTS assay, for their ability to reduce placental cell viability in vitro (Supplementary Fig. 1). From this, we identified vinorelbine as a potent inhibitor of cell viability. Given it is orally available, well tolerated and has potential to be translated into the clinic, we selected vinorelbine for further study.

Vinorelbine was administered to two human trophoblast (placental) cell lines, JEG3 cells (models cytotrophoblast cells) and HTR-8/SVneo cells (derived from human first trimester placenta and have characteristics of extravillous trophoblast cells) (Hannan et al., 2010; Mandl et al., 2006). We treated JEG3 or HTR-8/SVneo cells with increasing doses of vinorelbine (0.01-100 μM), as well as methotrexate (the only medical treatment for ectopic pregnancy in the clinic), and combination methotrexate and gefitinib (which is being evaluated in a large trial to treat ectopic pregnancy). At 100 μM, methotrexate did not significantly reduce cell viability in either placental cell type (Fig. 1A, B). Combination methotrexate (at 100 μM) and gefitinib (at 8 μM) significantly reduced cell viability in HTR-8/SVneo (Fig. 1B), but not JEG3 cells (Fig. 1A). In contrast, vinorelbine significantly reduced JEG3 and HTR-8/svneo cell viability in a dose-dependent manner (Fig. 1A, B), down to a concentration of 0.1 μM in JEG3 cells (1000 fold lower concentration than the methotrexate dose) and 1 μM in HTR-8/svneo cells. Furthermore, vinorelbine at 100 μM also proved more efficacious at inducing placental cell death than combination methotrexate (also at 100 μM) and gefitinib in both cell types.

We used flow cytometry to further quantify the proportion of live, apoptotic and dead HTR-8/SVneo cells after treatment. Reflecting the findings of our end point MTS assays, there was no significant difference in the proportions of live, apoptotic or dead cells after treatment with 100 μM of methotrexate (Fig. 1C–E). Combination methotrexate and gefitinib significantly reduced the proportion of live cells (Fig. 1C), but not the percentage of apoptotic or dead cells (Fig. 1D, E). In contrast, vinorelbine reduced the proportion of live cells from 0.1 μM concentration (1000 times lower than methotrexate) and increased apoptotic cells at a dose of 100 μM (Fig. 1C, D). There were trends toward increased proportions of dead cells with all drugs, though none were significant (Fig. 1E).

Next, we investigated the effects of vinorelbine on trophoblast proliferation using the xCELLigence system (Ke et al., 2011). Unlike endpoint assays, this measures cell proliferation in real-time by recording electrical impedance across wells. 12 h after treating with vinorelbine, there was significantly reduced cell proliferation in both JEG3 (Fig. 1F) and HTR-8/SVneo (Fig. 1G) cells at all concentrations administered (0.01-100 μM), compared to control. In contrast, neither methotrexate alone (at 100 μM), nor combination methotrexate and gefitinib induced a statistically significant decrease in cell proliferation at any time point after treatment (Fig. 1F, G). Of note, the administration of 0.01 μM vinorelbine to HTR-8/SVneo cells was significantly more efficacious than methotrexate at 100 μM (a concentration that is 10,000 times higher) in reducing proliferation between 20 and 30 h after treatment (Fig. 1G). Staining JEG3 and HTR-8/SVneo cells with the proliferation marker Ki67. Fig. 1h showed a reduction in Ki67 staining (as seen by green immunofluorescence) after 4 h of vinorelbine treatment at 1 μM, compared to control treated cells where an abundance of cell proliferation (Ki67 staining) is seen.

Together, our data thus far indicates that vinorelbine potently induces trophoblast cell death and inhibits proliferation in vitro at doses that are considerably lower than either methotrexate, or combination methotrexate and gefitinib.

Using placental explants we next examined the potential of vinorelbine to reduce primary first trimester placental tissue viability (Supplementary Table 1 details clinical characteristics). After treatment, we measured human chorionic gonadotrophin (hCG), a protein that is secreted into the circulation from the placenta and is a marker of placental cell viability (serum hCG levels are serially measured in the clinic to gauge the response to methotrexate when treating ectopic pregnancies either medically or expectantly, where a fall below 5mIU indicates complete resolution). Administering methotrexate at 100 μM ± gefitinib did not reduce hCG levels secreted into the culture media. In contrast, vinorelbine at 1 μM, (a concentration that is 100 times lower than methotrexate) significantly reduced hCG levels in the culture media (Fig. 1I).

3.2. Vinorelbine Potently Reduces Placental Xenografts In Vivo, and May be More Efficacious Than Methotrexate, or Combination Methotrexate and Gefitinib

To further our in-vitro results we examined the potential of vinorelbine to regress placental cells in vivo. We used a model that we previously described where JEG3 cells are xenografted subcutaneously into immune deficient (SCID) female mice and placental tumors allowed to form (Nilsson et al., 2013). Mice were then treated intravenously (via tail vein) with 1.25, 2.5 or 5 mg/kg vinorelbine, or vehicle control on days 6, 9 and 12 after xenograft inoculation. Compared to vehicle controls, vinorelbine at all doses significantly reduced xenograft volume 4 days after treatment (10 days after JEG3 inoculation) and each measured time point thereafter (12 and 14 days after inoculation) (Fig. 2A, B). Furthermore, vinorelbine treatment caused a significant, dose dependent reduction in xenograft tumor weight (harvested at the time of euthanasia) (Fig. 2C). There was a concordant dose dependent reduction in serum hCG from blood taken at the time of euthanasia. Significantly, we noted that at the top dose of vinorelbine (5 mg/kg) 6 out of 8 mice had a resolution of the tumor to the extent that no tumor could be found (Fig. 2C). Of these 6 mice, 5 had serum hCG levels below the detectable range of the assay (Fig. 2D). This suggests the possible complete absence of placental xenograft tumors in 5 mice.

Using the same model, we compared vinorelbine versus methotrexate alone, and methotrexate with gefitinib. When compared against vehicle control, methotrexate alone (at 2 mg/kg) or methotrexate with gefitinib (at 25 mg/kg) did not reduce placental tumor volume at any measured time point (Fig. 2e, f), tumor weight (Fig. 2g) or serum hCG levels at the time of sacrifice (Fig. 2h). In a separate experiment, we found methotrexate at 0.625, 1.25 and 2.5 mg/kg did not significantly decrease xenograft volume, weight or serum hCG (Supplementary Fig. 2).

In contrast to methotrexate, vinorelbine at 2.5 mg/kg reduced placental tumor volume at every time point from 2 days after treatment, (8 days after JEG3 inoculation) and each day thereafter (Fig. 2E, F). Tumor weights (Fig. 2G) and serum hCG (Fig. 2H) were also significantly reduced with vinorelbine treatment. In direct statistical

Fig. 3. Vinorelbine causes microtubule condensation, arresting trophoblast cell cycle and leading to activation of apoptosis. (A) Microtubules of JEG3 and HTR-8/SVneo cells after treatment with vinorelbine or control equivalent for 16 h, stained with α-tubulin (green) and DAPI (blue). Scale bar = 10 μm. (B–E) HTR-8/SVneo cell cycle phases after treating with vinorelbine, measured by intracellular staining of propidium iodide-A and FACs. (B) Representative histograms showing control treated cells with the proportions of cells in G_0/G_1 (green), S (yellow) and phase (purple). Treating with vinorelbine at (C) 1 μM and (d) 10 μM halts cell cycle progression at G_2/M, reducing the proportion of cells in S and G_0/G_1 phases. (E) Quantification of relative proportion of cells in G_0/G_1, S and G_2/M phases after vinorelbine treatment. Data are means ± SEM of three independent experiments in triplicate. (F–H) Western blot analysis showing increased protein expression of apoptotic markers after treating JEG3 and HTR-8/SVneo cells with vinorelbine. (F) Representative western blots of JEG3 (left) and HTR-8/SVneo (right) protein expression of Bcl-2, BAX, BNIP3 and caspase 9 and 3. Densitometric analysis of apoptotic markers in (G) JEG3 and (h) HTR-8/SVneo cells. Data are means ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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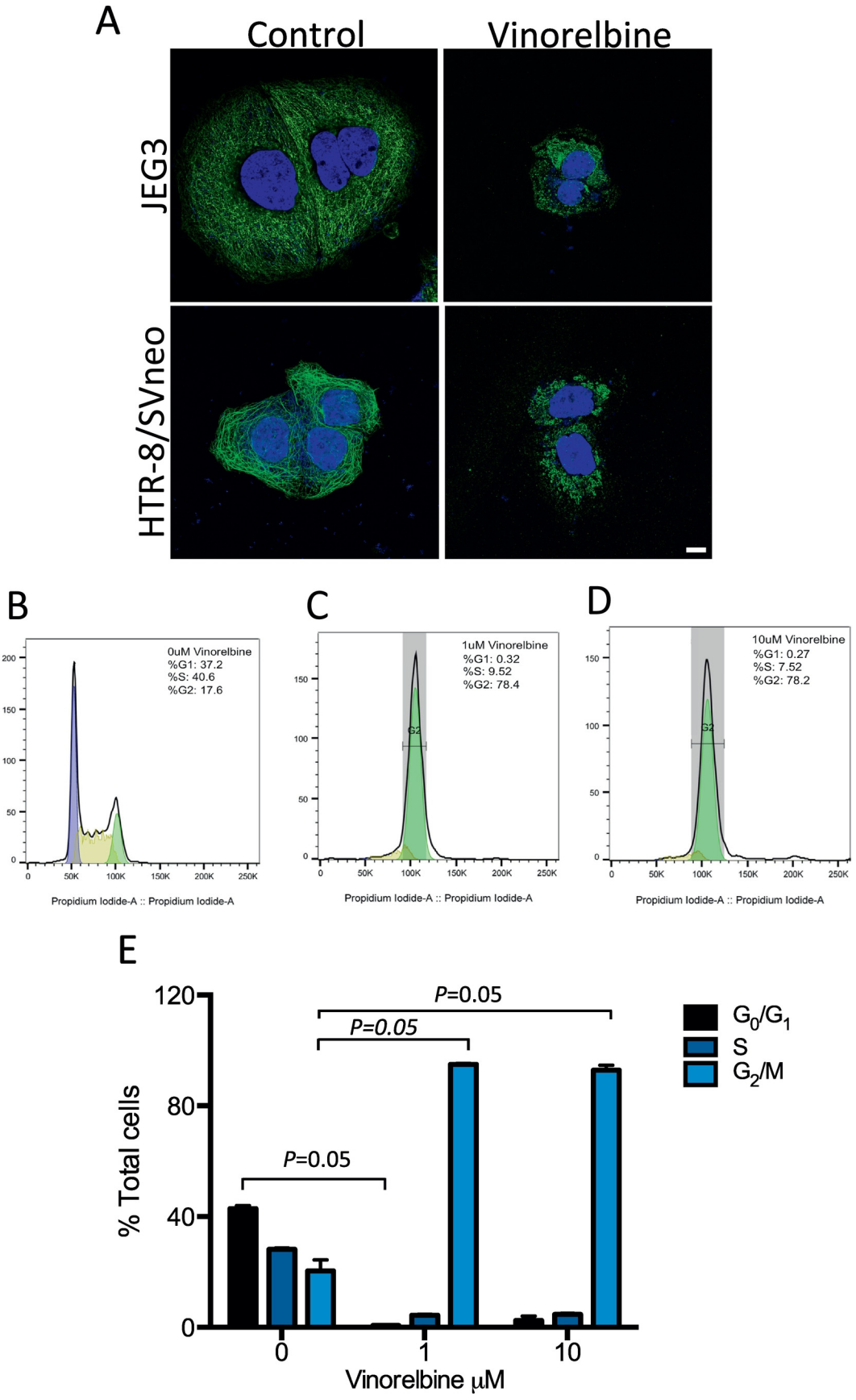
comparisons, vinorelbine was significantly more effective at reducing tumor volume than either methotrexate or combination methotrexate and gefitinib from days 8 and 11 respectively (Fig. 2E, F), more effective in decreasing xenograft tumor weight compared to methotrexate (Fig. 2G), and was associated with lower serum hCG compared to all other treatments (Fig. 2H).



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Our data suggests that vinorelbine potently reduces placental xenografts in vivo, and may be more efficacious than either methotrexate, or combination methotrexate and gefitinib.

3.3. Vinorelbine Causes Microtubule Condensation, Blocks Mitosis and Activates Apoptosis Cascades in Placental Cells

Vinorelbine is thought to cause cell death by binding β-tubulin, which depolarizes microtubulin, arrests cells in the metaphase to anaphase transition and ultimately leads to apoptosis of dividing cells (Okouneva et al., 2003). Hence, we examined whether this occurred in JEG3 and HTR-8/SVneo cells. After treating cells with vinorelbine, we stained them for the presence of tubulin, using confocal microscopy to visualize microtubulin. Administration of vinorelbine at 0.01 μM showed a notable reduction in microtubule staining (green fluorescence) in both cell types, compared to control. Additionally, the remaining tubulin appeared disordered and condensed (Fig. 3A). Given this condensation is thought to induce cell cycle arrest we next performed flow cytometry to measure cell cycle progression in HTR-8/SVneo cells treated with vinorelbine. As expected, vinorelbine caused a significant G_2/M block that coincided with a decrease in the proportion of cells in G_0/G_1 and S phase (Fig. 3E). This is visualized by representative histograms (Fig. 3B), where control treated cells show three distinct phases G_0/G_1 (purple), S (yellow) and G_2/M (green). However, histograms of vinorelbine treated cells show a reduction in the percentage of cells in G_0/G_1 and S phases and an increase G_2/M, indicative of a G_2/M mitosis block (Fig. 3c, d). The reduction of cells in G_0/G_1 and the increase in G_2/M with vinorelbine treatment was statistically significant (Fig. 3E).

In order to determine whether the cell cycle arrest caused by vinorelbine activated apoptotic cascades in both trophoblast cell types, we next measured protein expression of apoptotic genes following vinorelbine treatment. In both cell types, vinorelbine treatment significantly reduced Bcl-2 expression (apoptosis inhibitor; Fig. 3F–G). In contrast, we found vinorelbine treatment increased expression of the pro-apoptotic markers BAX, BNIP3 and cleaved caspases 3 and 9 (Fig. 3F–G). This suggests vinorelbine induces apoptosis in JEG3 and HTR-8/SVneo cells and this may be the mechanism of placental cell death.

Collectively, our data indicates vinorelbine induces placental cell death by condensing microtubules, leading to an expected G_2/M mitotic block and the activation of apoptotic cascades.

3.4. Vinorelbine Does Not Up-Regulate pro-Apoptotic Molecules in Human Fallopian Tubes and Does Not Affect Subsequent Fertility in Mice

Fertility preservation is major consideration for any potential treatment for ectopic pregnancy as women diagnosed with this condition are of reproductive age. It is possible vinorelbine could sparing the relatively quiescent Fallopian tube cells. We exposed human Fallopian tube explants obtained at hysterectomy from premenopausal women to vinorelbine (0.01–100 μM) for 48 h (Table S2 details the clinical characteristics). We then measured protein expression of the same apoptotic markers that we have shown to be activated by vinorelbine in placental cells. Vinorelbine did not increase expression of the pro-apoptotic molecules Bax, BNIP3, cytochrome *c* and caspases 3 and 9 (Fig. 4A, B) or reduce the expression of the apoptosis inhibitor Bcl-2 (Fig. 4A, B) in human Fallopian tubes. Thus, vinorelbine does not appear to induce apoptosis in human Fallopian explants. While it is likely the presence of an ectopic pregnancy itself may injure the Fallopian tube by causing scarring (de Bennetot et al., 2012), our data provides reassurance that vinorelbine does not seem to further induce cell death.

We next examined whether exposing mice to vinorelbine does affects future fertility. Female mice of reproductive age were exposed to three doses of vinorelbine at 5 mg/kg (the top dose used in our xenograft model) or vehicle control. After a 4-week wash out period (where the mice will have had 3–4 estrous (or menstrual) cycles) mice were time mated and euthanized at embryonic day 17.5. There were no differences between mice exposed to vinorelbine and controls in the number of pups per litter (Fig. 4C), pup or placental weight (Fig. 4D, E), crown to rump length (Fig. 4F), fetal placental ratio (Fig. 4G) or gross fetal morphology (Fig. 4I). Maternal serum anti-mullerian hormone (AMH) is measured clinically as an indicator of ovarian reserve. Serum AMH concentrations in mice were no different in the vinorelbine treated group, compared to controls (Fig. 4H) suggesting vinorelbine does not adversely affect the follicle pool. Vinorelbine does not appear to adversely affect subsequent fertility, breeding outcomes, or ovarian reserve.

4. Discussion

Here, we demonstrate that vinorelbine, an orally available and well-tolerated agent, has potential as a medical treatment for ectopic pregnancy. It appears to be highly active inducing placental cell death at concentrations that are 100–1000 fold lower than methotrexate (the only medical treatment used clinically), potently inhibits xenograft placental growth, does not induce pro-apoptosis genes in human Fallopian tubes, and does not impair subsequent fertility.

We first identified vinorelbine through screening of several chemotherapeutic agents where vinorelbine proved to be highly potent in reducing placental cell viability in both JEG3 and HTR-8/SVneo trophoblast cells. Surprisingly, vinorelbine induced placental cell death at doses that were 100 to 1000 times lower than methotrexate. Furthermore, vinorelbine reduced placental cell viability and proliferation beyond that of the combination of methotrexate (administered at the same dose of vinorelbine alone in the same experiment) and gefitinib. We next administered vinorelbine in a mouse model of ectopic pregnancy. In these in vivo experiments, the administration of vinorelbine at the top dose, 5 mg/kg, completely resolved placental cell mass in 6 out of 8 mice, with no palpable tumors and undetectable serum hCG concentrations (at the time of sacrifice) in 5 out of 8 mice. In subsequent experiments, directly comparing vinorelbine with methotrexate, and combination methotrexate and gefitinib, vinorelbine alone (at 2.5 mg/kg) proved significantly more efficacious than either treatment at resolving placental xenografts.

We have utilized a placental xenograft model, which was previously used in the investigation of combination methotrexate and gefitinib for ectopic pregnancy therapeutics (Nilsson et al., 2013). We note that this in vivo model does not fully recapitulate an ectopic pregnancy and the placental trophoblast tissue is not inoculated into the reproductive tract. However, such an animal model of ectopic pregnancy has not been described. Thus, we used an approach that has been widely used to evaluate drugs in oncology. Nevertheless we believe our model still provides highly informative data suggesting vinorelbine is able to successfully target human trophoblast tissues in vivo, and is far more efficacious than methotrexate (the only drug used to treat ectopic pregnancy in the clinic).

We note that in preclinical studies evaluating the use of vinorelbine in malignancies, the dose typically administered in xenograft models is 20 mg/kg (Villena-Heinsen et al., 1998; Hill et al., 1999; Dal Bello et al., 2015), four times higher than the highest dose used in our xenograft studies. Thus, it is possible that trophoblast cells may be particularly sensitive to vinorelbine in vivo, as well as in vitro. Furthermore, unlike malignancies, ectopic pregnancy tissues will have far less genetic mutations and resolve quicker than tumors, meaning acquired resistance to treatment should not arise. Thus, if vinorelbine is evaluated in clinical trials, dosing and dosing regimes could be substantially lower than that used to treat cancer.

Additionally, vinorelbine oral formulations are routinely administered in humans at 60 mg/m^2 (Depierre et al., 2001) and intravenously at 30 mg/m^2 (Depierre et al., 1991; Fumoleau et al., 1993; Hirsh et al., 2007). Extrapolating using FDA guidelines (Food Drug Administration, 2005) shows that administration of 2.5 mg/kg vinorelbine in a mouse to be 7.4 mg/m^2 human equivalent dose, approximately one quarter of

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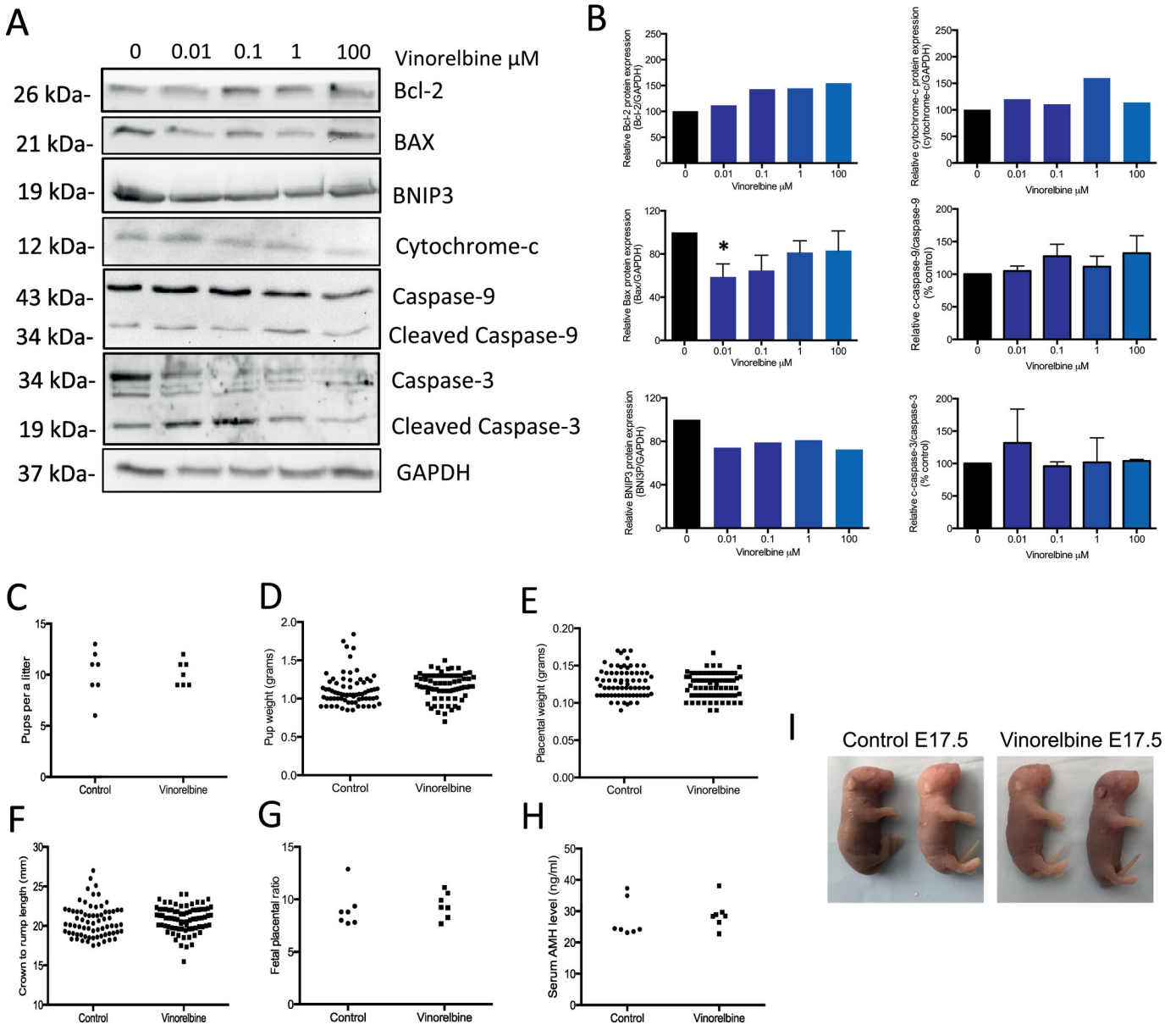

Fig. 4. Vinorelbine does not up-regulate pro-apoptotic molecules in human Fallopian tubes and does not affect subsequent fertility in mice. (A–G) Human fallopian tubes were collected from premenopausal women at the time of hysterectomy and treated with increasing doses of vinorelbine (0.01, 0.1, 1, 100 μM) or equivalent PBS for 24 h. (A) Representative western blot analysis performed for apoptotic markers Bcl-2, BAX, BNIP3, cytochrome *c* and caspase 9 and 3. (B) Densitometric analysis was performed on n = 8 samples for each apoptotic marker. The pro-apoptotic marker Bax was reduced with 0.01 μM vinorelbine. (C-I) Female swiss mice intravenously treated with vinorelbine 5 mg/kg or equivalent PBS, three times over two weeks. Four weeks post final treatment, mice were mated and then culled at embryonic day 17.5, pups, placenta and maternal blood were collected. (C) Litter size, (d) pup weight, (E) placental weight, (F) crown to rump length, (G) fetal placental ratio, (H) maternal serum levels of anti-mullerian hormone and (i) gross fetal morphology did not differ between control and treated groups (n = 7 per a group, no difference in vinorelbine or control treated by un-paired *t*-test). Data are means ± SEM. *p < 0.05 by 1-way ANOVA with Tukeys Test, comparing doses of vinorelbine treatment with vehicle control treatment.

the average intravenous dose of 30 mg/m^2. Thus, the doses we examined in our in vivo model were directly clinically relevant. Furthermore, with over 390 registered phase I to IV clinical trials assessing vinorelbine as a single agent or in combination treatment, robust information exists reporting the safety and tolerability of this chemotherapeutic (Marty et al., 2001; Gebbia and Puozzo, 2005; Depierre et al., 2001). Although neutropenia is the dose limiting toxicity for this chemotherapeutic, this is often only seen with prolonged treatment (Gebbia and Puozzo, 2005). Of other toxicities reported, including diarrhea, nausea and vomiting, these are seldom severe and usually respond to treatment (Depierre et al., 2001; Gregory and Smith, 2000). Additionally, metronomic vinorelbine (dense, nonbreak, low dose administration) is safely used and well tolerated, with some instances of treatment continuing for over 3 years without overt toxicity (Briasoulis et al., 2009; Camerini et al., 2015; Bilir et al., 2017). Furthermore, one report of 62 patients treated with metronomic vinorelbine (20–70 mg thrice weekly) reported non-hematological toxicities to be practically absent, describing vomiting and nausea as negligible (Briasoulis et al., 2009). Therefore, we believe if vinorelbine is translated into clinical use for ectopic pregnancy, due to the short treatment period and potential low dosing, toxicities related to vinorelbine may be mitigated.

Ongoing fertility is likely to be an important consideration for women diagnosed with an ectopic pregnancy when weighing up their management options. To address this potential concern, we exposed mice to the top dose of vinorelbine, which was highly effective in regressing the placental xenografts (5 mg/kg), and assessed subsequent fertility. Reassuringly, vinorelbine did not appear to affect litter size, the pups, or placental weight. Levels of serum anti-mullerian hormone

levels (a marker of ovarian reserve) were no different to controls. Although longer-term fertility outcomes where not assessed here, we are reassured by epidemiological reports of successful pregnancy outcomes following vinca-alkaloid treatment in women of reproductive age (Hodgson et al., 2007; Gershenson, 1988). Although reporting is limited on fertility outcomes following vinorelbine treatment as a drug class vinca-alkaloids are considered low risk for inducing gonadal toxicity (Sonmezer and Oktay, 2004; Shamberger et al., 1981). In support of our preclinical data on fertility, we note that reassuring human fertility data exists for vincristine, another vinca-alkaloid. In women with choriocarcinoma, treated with a chemotherapy regimen including vincristine but chose to retain their uterus: all resumed menses (100%), had successful pregnancies and did not appear to result in early menopause (Wong et al., 2014). In another study, there was no increase in chemotherapy-induced amenorrhoea (cessation of periods) among women treated with high dose vinorelbine (Zhou et al., 2010). Rat studies performed by others have shown vinorelbine is embryotoxic in low doses, however rats exposed to vinorelbine remain fertile (Pierre Fabre Mèdicament Production, 2002).

Our data has strong potential clinical implications. With only 30% of diagnosed ectopic pregnancies estimated to be eligible for methotrexate treatment (Jurkovic and Wilkinson, 2011) there is currently a large treatment bias towards surgery for this condition. It is therefore plausible that vinorelbine, which has proved more efficacious at lower doses than methotrexate in our pre-clinical work, could successfully treat larger ectopic pregnancies and expand the number of ectopic pregnancies that can be treated medically. Together our data here provide strong evidence to justify clinical trials to determine the efficacy of oral vinorelbine to treat ectopic pregnancy.

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Conflicts of Interest

ST, TKL and RH hold a patent (PCT/AU2016/051075) relating to the use of vinorelbine to treat ectopic pregnancy.

Author Contributions

RH, TKL and ST designed experiments and wrote the first draft of the manuscript. LC, PS, AH, EL, NH and FB provided intellectual input, technical support and contributed to the final manuscript.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.01.041.

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